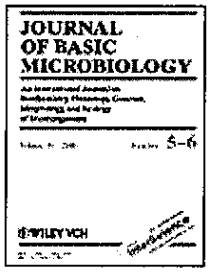

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Original Paper

Abundance of polymers degrading microorganisms in a sea-based solid waste disposal site

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Abstract

In order to assess the degradability of plastics in solid waste disposal landfill sites, microbial populations capable of degrading five kinds of plastic-constituting polymers, poly ε-caprolactone (PCL), poly-lactic acid (PLA), polyethylene glycol (PEG), poly-β-hydroxybutyrate (PHB) and cellulose acetate (CA), in a sea-based solid waste disposal site were investigated. Enumeration of aerobic and anaerobic polymers-degrading microorganisms (PDMs) was performed against to total 8 leachate samples, which were seasonally collected from the facultative pretreatment pond and the aerated lagoon. Both aerobic and anaerobic PDMs for natural polymers, PHB and CA, were found in all of the samples, while those for chemically-synthesized polymers, PCL, PLA and PEG, could not be always detected. In most cases, the ratios of the PHB- and CA-degraders to the heterotrophic bacterial population were more than 0.1%. On the other hand, the ratios of PCL-, PLA- and PEG-degraders were often much lower. These data indicate that the plastics degradation potential is commonly present in the studied disposal site, and that the degradation potential for plastics composed of chemically-synthesized polymers is inferior to that of natural polymers. Population sizes of the PDMs correlated to those of heterotrophic bacteria, and the counts of aerobic heterotrophic bacteria and PDMs in the aerated lagoon tended to be higher than those of anaerobic ones, indicating that the aeration of the leachate resulted in the activation of growth of whole aerobic microbial community including the PDMs.

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Abundance of polymers degrading microorganisms in a sea-based solid waste disposal site

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In order to assess the degradability of plastics in solid waste disposal landfill sites, microbial populations capable of degrading five kinds of plastic-constituting polymers, poly ε-caprolactone (PCL), polylactic acid (PLA), polyethylene glycol (PEG), poly-β-hydroxybutyrate (PHB) and cellulose acetate (CA), in a sea-based solid waste disposal site were investigated. Enumeration of aerobic and anaerobic polymers-degrading microorganisms (PDMs) was performed against to total 8 leachate samples, which were seasonally collected from the facultative pretreatment pond and the aerated lagoon. Both aerobic and anaerobic PDMs for natural polymers, PHB and CA, were found in all of the samples, while those for chemically-synthesized polymers, PCL, PLA and PEG, could not be always detected. In most cases, the ratios of the PHB- and CA-degraders to the heterotrophic bacterial population were more than 0.1%. On the other hand, the ratios of PCL-, PLA- and PEG-degraders were often much lower. These data indicate that the plastics degradation potential is commonly present in the studied disposal site, and that the degradation potential for plastics composed of chemically-synthesized polymers is inferior to that of natural polymers. Population sizes of the PDMs correlated to those of heterotrophic bacteria, and the counts of aerobic heterotrophic bacteria and PDMs in the aerated lagoon tended to be higher than those of anaerobic ones, indicating that the aeration of the leachate resulted in the activation of growth of whole aerobic microbial community including the PDMs.

The persistence of plastics in natural environments has been recognized as one of the greatest problems concerning waste disposal processes. It is not too much to say that the disposal of plastics is shortening the life of the landfill sites. As a countermeasure for such a problem, various biodegradable plastics have been developed and commercialized in recent years (SWIFT 1992). Theoretically they can constitute to prolongation of the lives of landfill sites. However, biodegradability of such plastics under in situ-conditions, especially in the landfill sites, has been poorly or scarcely understood until now, therefore, anyone cannot answer the question "would biodegradable plastics be actually degraded in the landfill sites and, accordingly, can they efficiently contribute to the waste reduction?" Thus, it is necessary to know more about the plastics degradation potential in landfill sites. From recent work it has been concluded that microorganisms capable of degrading polymer components might play a very important role in destruction of plastics (SCHIRMER *et al.* 1993, ISHIGAKI *et al.* 1999). This encouraged us to investigate the abundance of polymers-degrading microorganisms (PDMs) in a selected landfill site.

Here we report on the distribution of PDMs in a sea-based, municipal solid waste landfill site. Both aerobic and anaerobic PDMs capable of degrading selected plastics-constituting materials, i.e. poly-β-hydroxybutyrate (PHB), poly-ε-caprolactone (PCL), cellulose acetate (CA), polylactic acid (PLA) and polyethylene glycol (PEG) were enumerated. Heterotrophic bacterial flora in the same samples was also investigated, as they seem important as the background microbes of the PDMs.

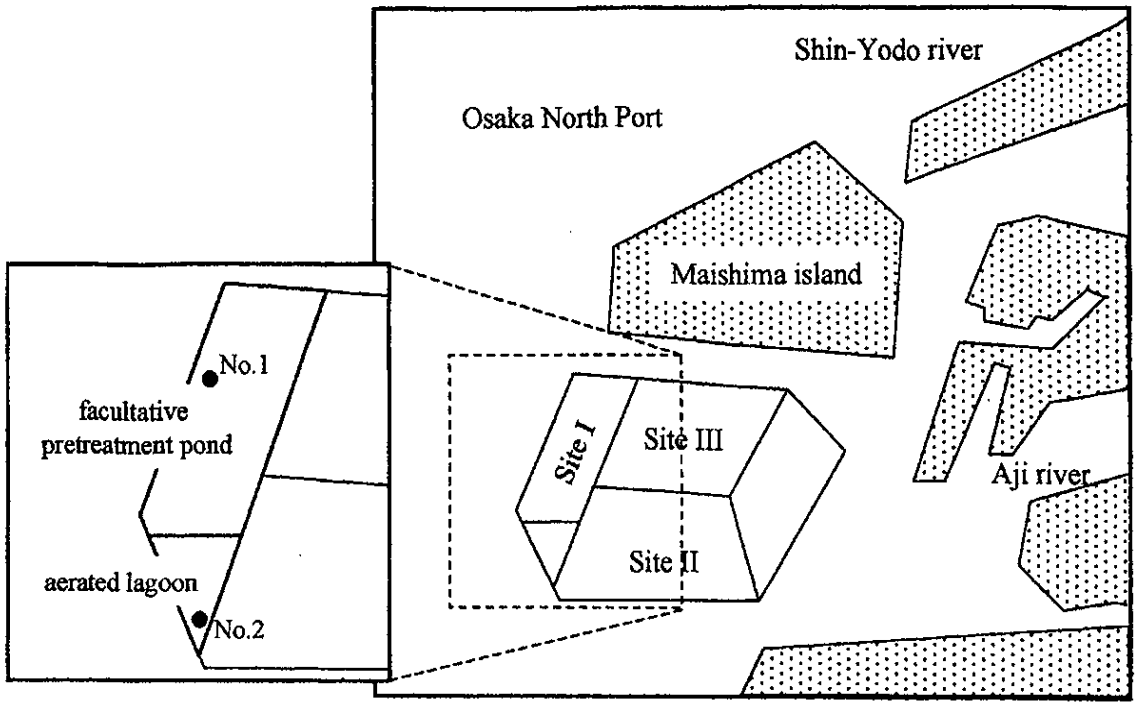


Fig. 1
Location of Osaka North Port sea-based waste disposal site

Materials and methods

Landfill site and sample collection: The leachate samples studied in this work were taken from the south section of Osaka North Port sea-based solid waste disposal site (FURUKAWA *et al.* 1994). Fig. 1 shows the location of the landfill site. The landfill consists of 3 sites, and only Site I is being used to be disposed of solid wastes. The area of Site I is 73 ha and consists of the facultative pretreatment pond (FPP) and the aerated lagoon (AL). The FPP is normal sea-based landfill site, being directly disposed of the wastes. On the other hand, the AL is mechanically aerated for reducing organic pollutants of the leachate from the FPP before being discharged into the open sea via Site II and III. The leachate samples were collected seasonally (4 times) from both sampling point No. 1 (FPP) and No. 2 (AL) between April 1997 to January 1998.

Polymers: PHB (ALDRICH Chemical Company Inc., molecular weight (Mw) average = 420000), PCL (WAKO Pure Chemical Co. Ltd., Mw av. = 10000), CA (ACROS Organics, Mw av. = 100000), PLA (WAKO Pure Chemical Co. Ltd., Mw av. = 5000) and PEG (KISHIDA Chemical Co. Ltd., Mw av. = 4000) were used as plastic-constituting polymers. PHB is polymer based on bacterially produced polyester. It is a bacterial storage material. CA is acetyl substitutes of cellulose, which is produced as a renewable resource from plant. These polymers were categorized to natural polymers (DEMICHELI 1996). On the other hand, PCL, PLA and PEG are made of petroleum-based polyester or polyether. Thus these polymers were categorized to chemically synthesized polymer.

Enumeration of PDMs: Agar plates containing the emulsified polymers were prepared by procedure described below, according to MORIKAWA *et al.* (1978) and AUGUSTA *et al.* (1993). 1000 mg of each polymer was dissolved in 20 ml of methylene chloride. Each solution was emulsified with a homogenizer (TOMY SEIKO Co. Ltd., Tokyo, Japan, UD-210, 10 min × 3) into 1000 ml of a basal medium (K_2HPO_4: 1000 mg/l, $(NH_4)_2SO_4$: 1000 mg/l, $MgSO_4 \cdot 7H_2O$: 200 mg/l, $FeCl_3$: 10 mg/l, NaCl: 50 mg/l, $CaCl_2$: 50 mg/l, yeast extract (DIFCO): 250 mg/l) containing 100 mg/l of TritonX-100. The medium was made using an artificial seawater (NaCl: 30 g/l, $MgCl_2 \cdot 6H_2O$: 10.8 g/l, $MgSO_4$: 5.4 g/l, KCl: 700 mg/l, $CaCl_2 \cdot 6H_2O$: 1.0 mg/l, $FeSO_4 \cdot 7H_2O$: 1.0 mg/l, $MnSO_4 \cdot 4H_2O$: 1.0 mg/l). Agar (1.5%, w/v)

was added to the emulsified medium and dissolved by heating, which simultaneously evaporated methylene chloride from the medium. Enumeration of the PDMs was performed using these emulsified polymer media by the plate count technique. The leachate samples were plated onto the media after appropriate dilution and they were cultivated at 28 °C for 10 days, according to NISHIDA *et al* (1993). Resultant colonies with halos, which indicate polymer degradation in the medium, were counted as PDMs. For enumerating aerobic and anaerobic PDMs, the plates were cultivated under ambient atmosphere and in an anaerobic incubator (TABAI ESPEC Co. Ltd., Osaka, Japan, EAN-140; $N_2 : CO_2 : H_2 = 90 : 5 : 5$), respectively.

Enumeration, isolation and taxonomical classification of heterotrophic bacteria: 1/4 PYG agar medium (bacto-peptone (DIFCO): 500 mg/l, yeast extract (DIFCO): 250 mg/l, glucose: 125 mg/l) was used for enumerating heterotrophic bacteria. The medium was prepared using the above-mentioned artificial seawater. The appropriately-diluted leachate samples were plated onto 3–5 plates and they were incubated at 28 °C for 5 days under either aerobic or anaerobic conditions. Colonies formed on the plates were counted as the aerobic and anaerobic heterotrophic bacteria, respectively. Almost all colonies formed on the plates were picked up, subcultured twice in 1/4 PYG liquid medium, and isolated as the pure cultures of representative bacteria present in the landfill leachate. The aerobic isolates were morphologically and physiologically characterized and identified up to genus level according to COWAN and STEEL (1973) and BERGEY's Manual (1984). Bacterial identification kits (API20NE and/or API20E; BIO MERIEUX S.A.) were also used for ensuring the identification. The anaerobic heterotrophic isolates were also morphologically and physiologically characterized and identified up to genus level according to Anaerobe Laboratory Manual (HOLDEMAN *et al.* 1977), based on the results of analyses of acetate, propionate, butyrate and lactate accumulated in the culture broth. The organic acids in the culture broth were analyzed using a HITACHI 263-50 type gas chromatography equipped with a FID with THERMON 3000 column.

Diversity index of bacterial flora: Simpson Index (SIMPSON 1949) expressed by the following equation was used to characterize the diversity of the heterotrophic bacterial community in the leachate samples.

$$\beta = \frac{N(N-1)}{\sum_i n_i (n_i - 1)}$$

N: the number of total heterotrophs isolated, n_i: the number of isolates of each genus or category.

Results

Physico-chemical conditions of the landfill site

Physico-chemical conditions of the 2 sampling points, FPP and AL, of the landfill site are summarized in Tables 1 and 2, respectively. The indices investigated here showed similar values for both sampling points throughout the experimental period except for dissolved oxygen (DO) concentration. The DO values in the FPP and AL were 1.4–1.6 and 3.9–9.4 mg/l, respectively, and seem to be maintained at relatively high levels as a sea-based landfill site

Table 1
Physico-chemical conditions at sampling point No. 1 (FPP)

	Spring (4/23/1997)	Summer (9/11/1997)	Autumn (11/7/1997)	Winter (1/14/1998)
Water temp. (°C)	14.2	28.5	17.5	6.0
pH	7.9	8.1	7.8	8.2
DO (mg/l)	1.5	1.4	1.6	1.4
BOD (mg/l)	12.4	5.6	5.0	5.6
COD (mg/l)	50.0	37.2	34.4	36.1

Table 2
Physico-chemical conditions at sampling point No. 2 (AL)

	Spring (4/23/1997)	Summer (9/11/1997)	Autumn (11/7/1997)	Winter (1/14/1998)
Water temp. (°C)	14.0	28.0	17.0	5.5
pH	7.9	8.2	7.9	8.2
DO (mg/l)	4.1	3.9	4.7	9.4
BOD (mg/l)	12.5	9.1	7.2	5.3
COD (mg/l)	52.0	35.6	33.5	36.4

(Japan Society of Civil Engineering, 1998). The water temperature varied from 5.5–6.0 °C at winter to 28.0–28.5 °C at summer. The concentrations of organic matter varied little within the ranges around 5–10 mg/l as BOD (biochemical oxygen demand) and 35–50 mg/l as COD (chemical oxygen demand), which were similar ranges observed in other sea-based landfill sites (Japan Society of Civil Engineering 1998).

Distribution of PDMs

Results of the enumeration of PDMs in the leachate samples from the FPP and AL are summarized in Tables 3 and 4, respectively, in comparison with the heterotrophic bacterial counts. Both aerobic and anaerobic PDMs for 5 kinds of polymers were detected from almost all samples. Especially, CA- and PHB-degraders were found from all the samples, and accounted for more than 0.1% of the heterotrophic populations in most cases. On the other hand, the spring sample from the FPP did not contain detectable numbers of aerobic PLA- and PEG- and anaerobic PEG-degraders, and the winter sample from the AL did not anaerobic PCL- and PEG-degraders. Further, the ratios of PCL-, PLA- and PEG-degraders were often much lower than 0.1%, even when they were detected.

Table 3
Results of the enumeration of heterotrophic bacteria and PDMs in the FPP samples (CFU/ml). Ratios of the PDMs population to the heterotrophic bacteria were also shown

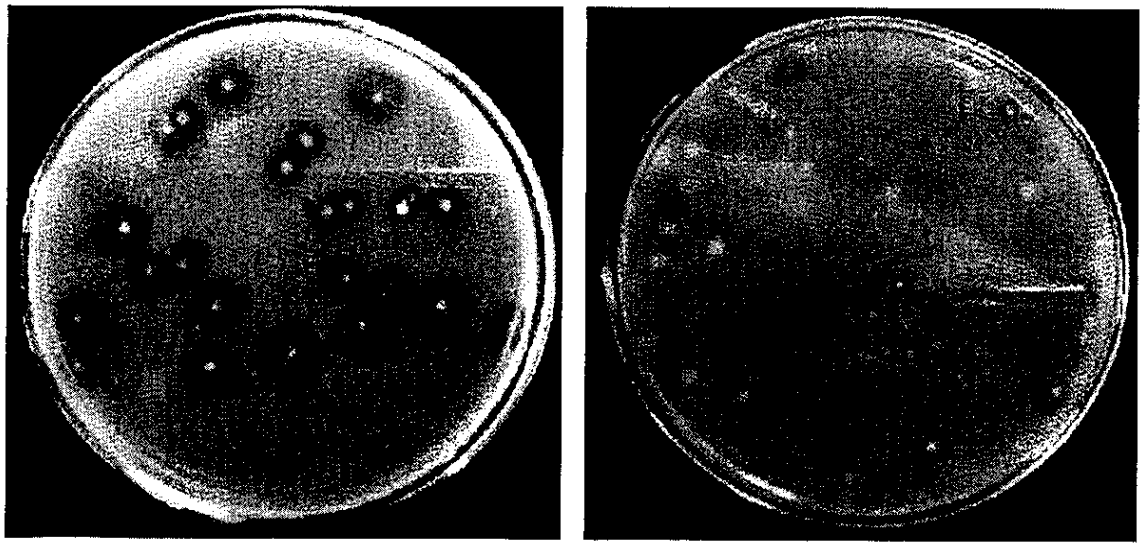
		hetero-troph	polymer degrader				
			CA	PHB	PCL	PLA	PEG
Spring	anaerobe	3.3×10^4	9.0×10^2 2.7%	2.8×10^3 8.5%	5.0×10^3 15%	7.5×10^3 23%	N.D. –
	aerobe	1.0×10^5	1.0×10^2 0.1%	3.8×10^3 3.8%	2.0×10^2 0.2%	N.D. –	N.D. –
Summer	anaerobe	1.4×10^6	1.6×10^4 1.1%	2.3×10^4 1.6%	1.0×10^4 0.7%	2.8×10^4 2.0%	1.2×10^4 0.9%
	aerobe	1.5×10^5	2.7×10^4 18%	2.5×10^4 17%	3.2×10^4 21%	3.9×10^4 26%	3.3×10^4 22%
Autumn	anaerobe	8.8×10^5	7.0×10^4 8.0%	2.5×10^5 28%	5.8×10^4 6.6%	8.0×10^2 0.09%	2.4×10^4 2.7%
	aerobe	6.5×10^4	6.7×10^1 0.1%	2.0×10^2 0.3%	6.6×10^1 0.1%	6.6×10^1 0.1%	3.3×10^1 0.05%
Winter	anaerobe	2.1×10^5	2.7×10^2 0.1%	7.9×10^2 0.4%	2.3×10^2 0.1%	6.2×10^2 0.3%	1.0×10^1 0.005%
	aerobe	8.8×10^4	3.5×10^2 0.4%	4.7×10^3 5.3%	1.5×10^2 0.2%	1.1×10^1 0.01%	7.4×10^2 0.8%

N.D.: not detected (less than 1 CFU/ml)

Table 4
Results of the enumeration of heterotrophic bacteria and PDMs in the AL samples (CFU/ml). Ratios of the PDMs population to the heterotrophic bacteria were also shown.

		hetero-troph	polymer degrader				
			CA	PHB	PCL	PLA	PEG
Spring	anaerobe	1.3×10^5	4.0×10^2 0.3%	3.4×10^4 26%	3.3×10^0 0.003%	6.0×10^2 0.5%	1.0×10^2 0.08%
	aerobe	5.4×10^5	1.0×10^4 1.9%	7.4×10^3 1.4%	8.0×10^2 0.2%	1.0×10^3 0.2%	1.0×10^3 0.2%
Summer	anaerobe	1.4×10^6	3.5×10^3 0.3%	6.1×10^3 0.4%	2.4×10^3 0.2%	8.0×10^2 0.06%	9.0×10^3 0.6%
	aerobe	5.8×10^5	4.1×10^4 7.1%	1.8×10^4 3.1%	5.6×10^4 9.7%	4.1×10^4 7.1%	2.8×10^4 4.8%
Autumn	anaerobe	2.1×10^4	1.0×10^2 0.5%	8.0×10^3 38%	1.0×10^2 0.5%	8.0×10^2 3.8%	2.3×10^2 1.1%
	aerobe	5.6×10^4	1.1×10^2 0.2%	1.5×10^2 0.3%	1.2×10^2 0.2%	2.0×10^2 0.4%	1.5×10^1 0.03%
Winter	anaerobe	3.4×10^4	2.0×10^1 0.06%	1.0×10^1 0.03%	N.D. –	2.0×10^1 0.06%	N.D. –
	aerobe	4.7×10^4	4.8×10^1 0.1%	1.1×10^2 0.2%	3.1×10^2 0.7%	2.9×10^2 0.6%	2.5×10^2 0.5%

N.D.: not detected (less than 1 CFU/ml)



A B

Fig. 2 A typical example of appearances of the colonies of (A) aerobic and (B) anaerobic PDMs. Figures shows the colonies and halos on the PCL emulsified plates

The population sizes of the PDMs seasonally fluctuated both in the FPP and AL samples considerably. The counts of aerobic PDMs tended to be higher than those of anaerobic PDMs in the AL samples, however, no such a tendency was observed in the FPP samples. Fig. 2 shows a typical example of the appearance of colonies on the PDM-counting media (the emulsified polymer media) after 10-day incubation. The halo formation by the aerobic and anaerobic PDMs is compared in the figure. The halo sizes, which seem to represent the degradation activity (MORIKAWA *et al.* 1978), formed by the aerobic PDMs are much larger than those formed by the anaerobic PDMs in general.

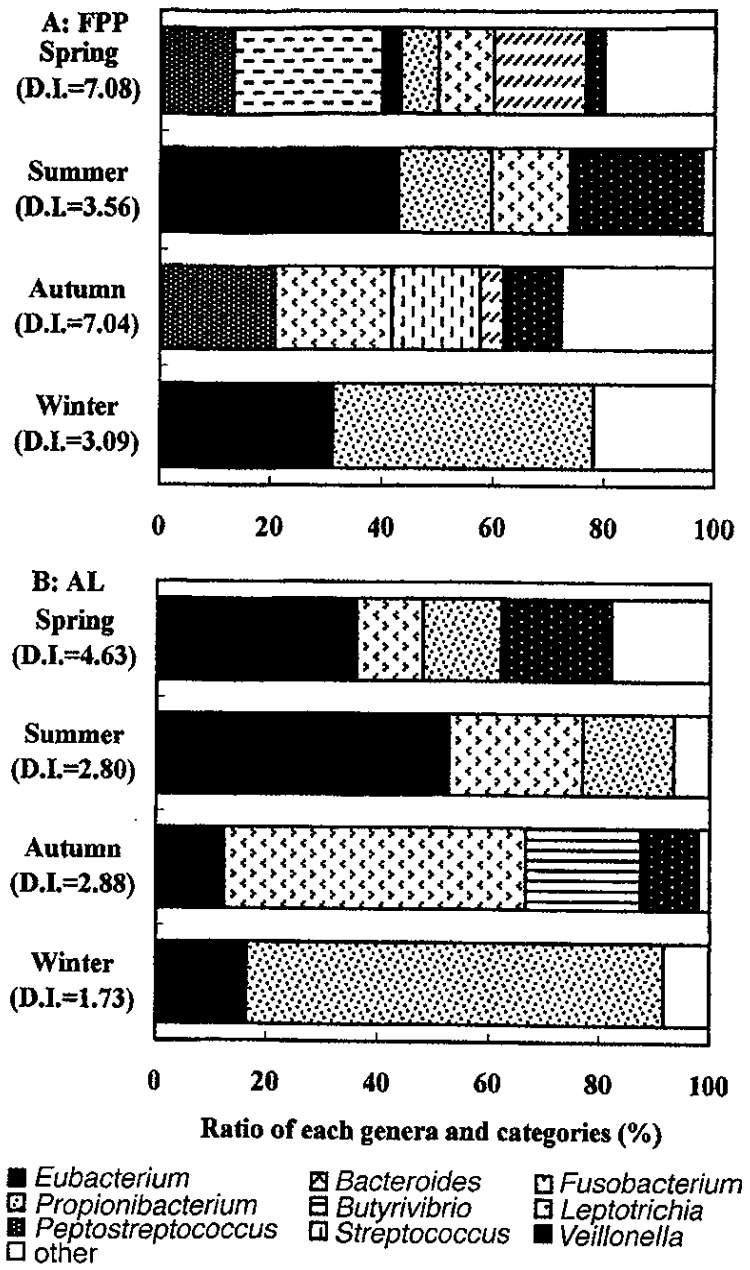


Fig. 3
Anaerobic bacterial flora in (A) FPP and (B) AL samples

Heterotrophic bacterial flora

Data on heterotrophic bacterial abundance in the FPP and AL samples are given in Tables 3 and 4, respectively. The aerobic heterotrophic bacterial counts ranged from 4.8×10^4 to 5.8×10^5 CFU/ml, while the anaerobic heterotrophs from 2.1×10^4 to 1.4×10^6 CFU/ml.

The dominant aerobic and anaerobic bacterial genera in the leachate samples are expressed as the ratios of bacterial genera or categories (e.g., Coryneforms) as shown in Figs. 3 and 4, respectively. Both the aerobic and anaerobic bacterial flora varied seasonally and between in the FPP and AL samples. The aerobic bacterial genera or categories commonly isolated from the FPP and AL samples were *Acinetobacter*, *Pseudomonas*, *Yersinia*, *Bacillus* and Coryneforms, while the anaerobic bacteria were *Eubacterium*, *Fusobacterium*, *Propionibacterium* and *Veillonella*. According to the calculation of Simpson index defined in

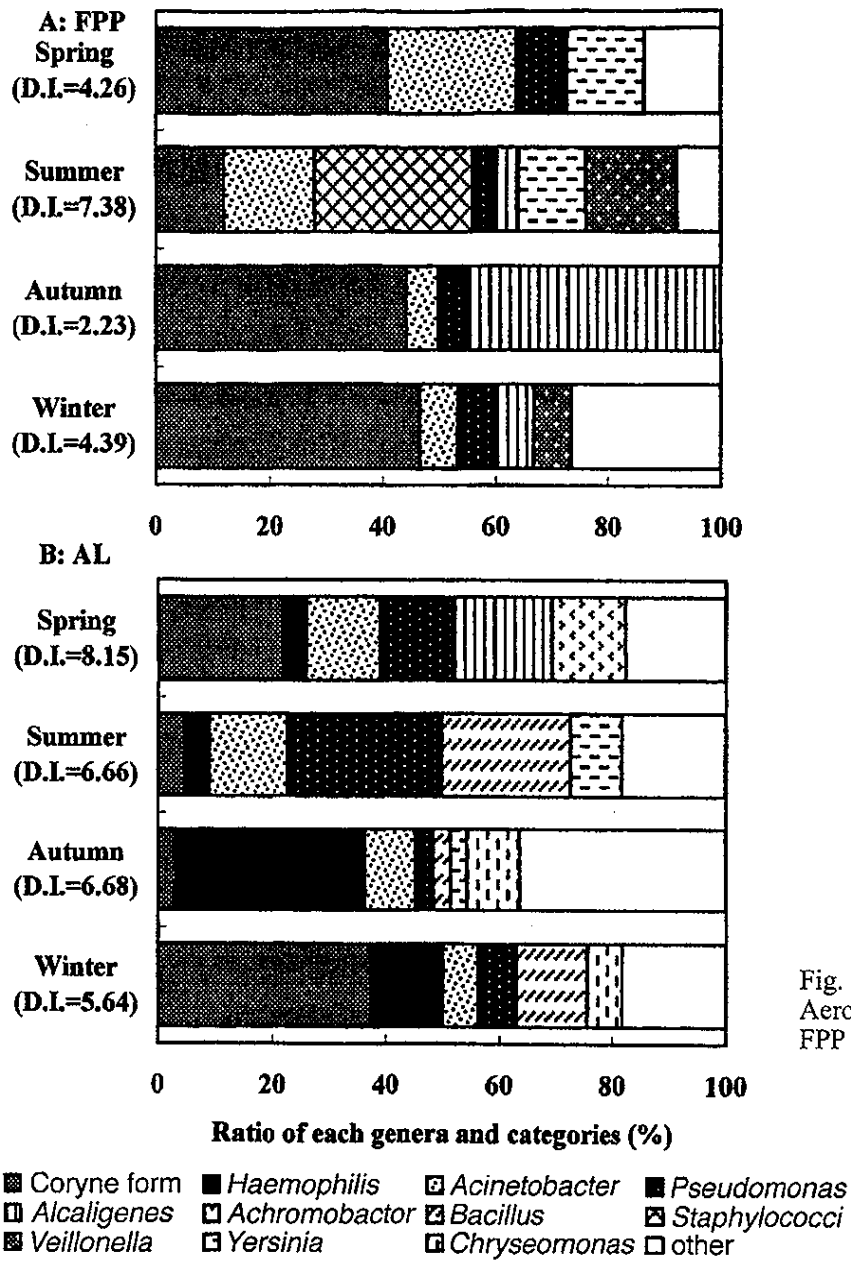


Fig. 4
Aerobic bacterial flora in (A) FPP and (B) AL samples

the previous part, diversity of aerobic bacterial flora was higher in the AL samples than in the FPP samples as a whole. On the other hand, the contrary trend was observed on the diversity of the anaerobic bacterial flora.

Correlation between the population sizes of PDMs and heterotrophic bacteria

Fig. 5 shows the relationship between the counts of PDMs and heterotrophic bacteria. As shown in the figure, the population of PDMs had a significant correlation with that of heterotrophs (correlation coefficient; R = 0.68). A higher correlation (R = 0.67) was observed between the counts of aerobic PDMs and heterotrophs than between anaerobic ones (R = 0.53). Although relationships between population sizes of the PDMs and other parameters, e.g., water temperature, DO, BOD, COD, diversity of heterotrophic bacterial flora etc., were also investigated, no significant correlation was found (data analyses not shown).

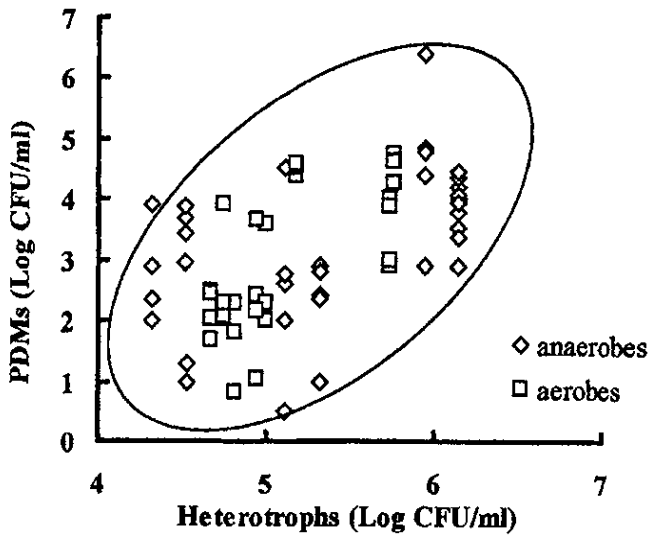


Fig. 5
Relationship between the population sizes of PDMs and heterotrophic bacteria

Discussion

Although the information on the abundance and/or distribution of PDMs in the natural environment is quite important for assessing the bigdegradability of plastics, it has been dealt with by only a few studies up to date (POTTS *et al.* 1972, NISHIDA *et al.* 1994). Especially concerning the PDMs in the landfill sites, there has been no report excepting only one paper, in which the presence of aerobic PCL- and PHB-degraders in 2 landfill leachate samples (from Tokyo Bay, Japan) was described (NISHIDA *et al.* 1994). Here we have extensively investigated the distribution of PDMs in the leachate from a sea-based landfill site for 5 kinds of plastic-constituting polymers.

PDMs for each polymer were detected at certain high levels from almost all samples, indicating ubiquitous and abundant presence of PDMs for various polymers in the landfill sites. In other words, plastic degradation potentials are commonly present in the landfill sites. Although PDMs for natural polymers, PHB and CA, were found from all the samples, those of chemically-synthesized polymers, PCL, PLA and PEG, could not be always detected. Further, PDMs for natural polymers accounted for higher percentages to the heterotrophic bacteria than PDMs for chemically-synthesized polymers as a whole. TORRES *et al.* (1996) also reported that only a few kinds of microorganisms could degrade PLA- and LA-containing polymers, and KAWAI (1989) reported that higher-molecular-weight PEG showed very low biodegradability. This suggests that the degradation potential of plastics composed of chemically-synthesized polymers in the natural environments including landfill sites is inferior compared to that of plastics composed of natural polymers.

In this study, the leachate samples collected from the FPP and AL were used for enumerating PDMs. In both locations, similar environmental conditions were observed except for the DO concentration. In the AL, where mechanical aeration created a very high DO level, populations of the aerobic PDMs and heterotrophs tended to be larger than the anaerobic ones, and the diversity of the aerobic heterotrophs was higher than in the FPP. Further, judging from the bacterial genera and/or categories shown in Fig. 3, percentages of the strict aerobes to the total aerobic heterotrophs were higher than in the FPP. These indicate that selective growth of aerobic bacteria including PDMs occurred in the AL. On the other hand, relative abundance and diversity of anaerobic PDMs and heterotrophs in the FPP were higher than in the AL, though the DO concentration in the FPP was maintained at relatively high levels. Since the aerobic PDMs possessed higher polymer degrading activities compared with the anaerobic PDMs judging from the halo sizes (Fig. 2), activation of

aerobic PDMs by the mechanical aeration of leachate might be an effective mean to accelerate the plastic degradation in the landfill sites.

The aerobic and anaerobic heterotrophic bacterial flora were also investigated as the background microbes of the PDMs, and a relatively high correlation between populations of PDMs and heterotrophs was shown, suggesting that the PDMs play a certain role in the microbial community. We reported here the dominant bacterial genera and/or categories and frequent transition of bacterial flora in landfill site. Some of the dominant aerobic heterotrophic bacteria found in this study were also reported by KAWAI *et al.* (1988). However, still little has been known, and further intensive studies are necessary to clarify the microbial ecology of the landfill sites.

From the results of this study, it may be concluded that certain degradation potentials of various kinds of biodegradable plastics exist in the landfill sites, and that the proper management or operation of the environmental conditions, e.g. mechanical aeration of the leachate, will lead to stimulation of PDMs. Strategies of the management or operation of the landfill sites as the bioreactors utilizing indigenous microbes have been already proposed by POHLAND *et al.* (1995) and BARLAZ *et al.* (1989). Several previous study have been concluded that polymers-degrading microorganisms would play a very important role in destruction of plastics (SCHIRMER *et al.* 1993, ISHIGAKI *et al.* 1999). Therefore, by applying the bioreactor landfill techniques, biodegradable plastics may show the enhanced degradability in waste landfill site.

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